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(54) Title: A METHOD OF SCREENING CELL POPULATIONS

(57) Abstract: A method of screening a large population of variant cells or cell colonies present on a first surface, which cells or colonies may be capable of producing a useful polypeptide, RNA or small molecule, which method comprises the steps of i) on the first surface, subjecting the cells or cell colonies to an assay correlated to a property of the useful polypeptide, RNA or small molecule; ii) by means of a colony picker, selecting cells having the property from the first surface; and iii) transferring the selected cells to a second surface.

A METHOD Of SCREENING CELL POPULATIONS

The present invention relates to a method of screening cell populations. More specifically, the invention relates to use of a colony picker in a pre-screening assay to search a large population of cells, especially DNA libraries, for the ability to produce substances of particular interest.

BACKGROUND OF THE INVENTION

Technologies such as DNA shuffling, random mutagenesis and in vivo recombination have allowed the generation of enormous populations of variant cells which produce variants of a certain protein, RNA, or small molecule. In addition, it has become possible to establish large DNA libraries of natural proteins from other organisms in production strains. Together, this has created a need to develop assays by which large numbers of variant cells can efficiently and accurately be screened. Also, automatization has become necessary to handle these large populations. One such automated device is a colony picker.

SUMMARY OF THE INVENTION

The inventors have now found a number of methodologies for using a colony picker for pre-screening DNA libraries in order to enrich for the more active variant cells.

Accordingly, the present invention provides a method for screening DNA libraries wherein colony picking is used as a means for selecting and transferring desired variant cells from a first surface to a second surface. Subsequently, the transferred variant cells may be subjected to an activity-based or application specific assay. Preferably, the assay is a microtiter plate-based assay. Usually, such an assay has a lower capacity, i.e. a smaller throughput, than the pre-screen/colony picking but is more specific in relation to the intended application of the protein or small molecule produced by the variant cell. Preferably, the cells transferred from the first surface is a colony.

Thus, in a first aspect the present invention relates to

a method of screening a large population of variant cells or cell colonies present on a first surface, which cells or colonies may be capable of producing a useful polypeptide, RNA or small molecule, which method comprises the steps of i) on the first surface, subjecting the cells or cell colonies to an assay correlated to a property of the useful polypeptide, RNA or small molecule; ii) by means of a colony picker, selecting cells having the property from the first surface; and iii) transferring the selected cells to a second surface.

By using the method of the present invention is has now been made feasible to pre-screen very large populations of variant cells or cell colonies for material of interest, e.g. for polypeptides/proteins, RNA or small molecules which are produced by the cells or colonies and which has useful and desirable properties. An example is prescreening of DNA libraries for an industrially useful enzyme having high enzymatic activity in the alkaline pH range.

BRIEF DESCRIPTION OF THE DRAWINGS

A protease depleted *B. subtilis* strain (SHA273) was transformed with either no DNA or a protease (Savinase) expressing plasmid (pSX222). An overnight culture of SHA273 and pSX222/SHA273 was individually diluted to a density of $1x10^3$ cells/ml and plated on agar plates with increasing concentrations of the antimicrobial peptide Protamine (0, 50, 75, 100, 125. 150, 175, 200 μg/ml). Agar plates were incubated overnight at 37 °C and the number of colonies were scored.

The result is plotted in figure 1 and shows that Protamine inhibits the growth of cells with increasing concentrations. In addition, cells expressing Savinase has a higher survival rate compared to cells expressing no Savinase, in the presence of Protamine.

DETAILED DESCRIPTION OF THE INVENTION

The colony picker

In the present context, the term "colony picker" denotes an apparatus capable of a) detecting desirable cells or

colonies present on a first surface by means of an automated visual analysis of the cells or colonies which is based on predetermined criteria; and b) transferring the desired cells or colonies from the first surface to a second surface. The first and second surfaces are preferably a plate, optionally a substrate plate including substrate for growth or non-growth of the desired variant cells or colonies. The plate may be a well plate or a plate with variant cells or colonies arranged in a spatial array. The colony picker produces a digital picture of the plate including the cells or colonies and analyses the picture in order to locate the desired cells or colonies which may be detected as being e.g. coloured, growing, growth-inhibited, colourless or fluorescent. Based on the detection of location of the desired variant cells or colonies, the colony picker touches each desired variant cell or colony with a needle in order to transfer material from the colony to the needle and the needle then transfers the material to a second surface, for example to inoculate growth media in a microtiter well. Before the next colony is picked the needle is sterilized, for example in a bath containing ethanol or another conventional sterilizing chemical compound or composition.

A useful colony picker is the 'Q' Pix Colony Picker which is an automated benchtop Colony Picker, Gridder and MicroArrayer manufactured by Genetix. The 'Q' Pix colony picker can pick and re-array over 3.500 colonies per hour into 384 or 96 well plates. It may be set to pick based on absorption at a given wavelength. It picks out of 22x22 Bioassay trays, Petri Dishes or Omnitrays. When picking colonies, the needles are sterilized in sterilization baths after a colony has been picked, and before the next colony is picked, by forcing sterilization solutions (e.g. ethanol) across the pins at high pressure. The colony picker may also be used to pick from 384 or 96 well plates into agar plates (called gridding). Arrays are done with a 16-Pin Head, using either for example 0.15 um diameter Genetix solid pins, or much thicker needles, for example with a diameter of 1 mm.

Other useful colony pickers are devices capable of picking based on any visual detection of desirable variant

cells or colonies, i.e. light emission or light absorption including fluorescence.

Definitions

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989") *DNA Cloning: A Practical Approach*, Volumes I and II /D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds (1985)); *Transcription And Translation* (B.D. Hames & S.J. Higgins, eds. (1984)); *Animal Cell Culture* (R.I. Freshney, ed. (1986)); *Immobilized Cells And Enzymes* (IRL Press, (1986)); B. Perbal, *A Practical Guide To Molecular Cloning* (1984).

When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the proteins in a highly purified form, i.e., greater than 95% pure, more preferably greater than 99% pure. When applied to a polynucleotide molecule, the term "isolated" indicates that the molecule is removed from its natural genetic milieu, and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules are free of other genes with which they are ordinarily associated, and may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316: 774-78, 1985).

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

The term "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary or quaternary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

The term DNA "coding sequence" denotes a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

The variant or host cells or variant or host colonies

The present invention relates to screening of variant or host cells and recombinant variant or host cells comprising a DNA sequence of interest or nucleic acid sequence that is advantageously used in the recombinant production of the material of interest. The term "variant cell" or "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a nucleic acid sequence followed by integration of the vector into the host chromosome.

Transformation means introducing a vector comprising a nucleic acid sequence into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome may occur by homologous or non-homologous recombination as described above.

The choice of a variant or host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The host cell may be a unicellular microorganism, *e.g.*, a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Useful unicellular cells are bacterial cells such as gram-positive bacteria including, but not limited to, a *Bacillus* cell, *e.g. Bacillus alkalophilus, Bacillus agaradhaerens, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus clausii, Bacillus circulans, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus stearothermophilus, Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g., Streptomyces lividans* or *Streptomyces murinus*, or gram-negative bacteria such as *E. coli* and *Pseudomonas* sp.

The transformation of a bacterial variant or host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168:111-115), by using competent cells (see, *e.g.*, Young and

Spizizin, 1961, *Journal of Bacteriology* 81:823-829, or Dubnar and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56:209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6:742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169:5771-5278).

The variant or host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or a fungal cell.

Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, 10314 and 1573, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., N.Y., 1987, Hawley-Nelson et al., Focus 15 (1993), 73; Ciccarone et al., Focus 15 (1993), 80; Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

The variant or host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In*, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella* (=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and

aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water moulds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

A fungal variant or host cell may also be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (*e.g.*, genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (*e.g.*, genera *Pichia*, *Kluyveromyces* and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti is divided into two families, Sporobolomycetaceae (*e.g.*, genera *Sorobolomyces* and *Bullera*) and Cryptococcaceae (*e.g.*, genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics are well known in the art (see, *e.g.*, *Biochemistry and Genetics of Yeast*, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; *The Yeasts*, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.*, editors, 1981).

The yeast variant or host cell may be selected from a cell of a species of *Candida*, *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Pichia*, *Hansehula*, , or *Yarrowia*. Useful yeast host cells are *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. Other useful yeast host cells are a *Kluyveromyces lactis* *Kluyveromyces fragilis* *Hansehula polymorpha*, *Pichia pastoris* *Yarrowia lipolytica*,

Schizosaccharomyces pombe, Ustilgo maylis, Candida maltose, Pichia guillermondii and Pichia methanolio cell (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279 and US 4,879,231).

The fungal variant or host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. The filamentous fungal host cell can be a cell of a species of, but not limited to, *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Thielavia, Tolypocladium,* and *Trichoderma* or a teleomorph or synonym thereof.

Particularly useful filamentous fungal variant or host cells are *Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus niger, Aspergillus nidulans* or *Aspergillus oryzae*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78: 147-156. Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae, A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023.

Yeast may be transformed using the procedures described

by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153:163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:1920. Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, *Virology* 52:546).

Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4,775,624; US 4,879,236; US 5,155,037; US 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

A recombinant vector into which DNA (coding for a desired polypeptide produced by the variant or host cell) is inserted may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the desired polypeptide is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence that shows

transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the polypeptide of the invention in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809 - 814) or the adenovirus 2 major late promoter. An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., FEBS Lett. 311, (1992) 7 - 11), the P10 promoter (J.M. Vlak et al., J. Gen. Virology 69, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus variant or host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral a-amylase, *A. niger* acid stable a-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

Examples of suitable promoters for use in bacterial variant or host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus*

licheniformis alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gen, or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda P_R or P_L promoters or the E. coli lac, trp or tac promoters.

The DNA may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the variant or host cell in question. An example of such a sequence (when the variant or host cell is a mammalian cell) is the SV40 origin of replication.

When the variant or host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2m replication genes REP 1-3 and origin of replication.

When the variant or host cell is a bacterial cell, sequences enabling the vector to replicate are *legio* in the art.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, and sC.

To direct a polypeptide encoded by the DNA into the secretory pathway of the variant or host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA

sequence encoding the polypeptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide. The secretory signal sequence may be that normally associated with the polypeptide or may be from a gene encoding another secreted protein.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide ensuring efficient direction of the expressed polypeptide into the secretory pathway of the cell. The signal peptide may be a naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the a-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the polypeptide. The function of the leader peptide is to allow the expressed polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the polypeptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast a-factor leader (the use of which is described in e.g. US 4,546,082, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease, or a *Humicola lanuginosa* lipase. The signal

peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral a-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase.

For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

The procedures used to ligate the DNA, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

It is also within the scope of the present invention to employ transgenic animal technology to produce the variant cells or the polypeptide or small molecule of interest. A transgenic animal is one in whose genome a heterologous DNA sequence has been introduced. In particular, a polypeptide of the invention may be expressed in the mammary glands of a non-human female mammal, in particular one which is known to produce large quantities of milk. Examples of preferred mammals are livestock animals such as goats, sheep and cattle, although smaller mammals such as mice, rabbits or rats may also be employed.

The DNA sequence of interest may be introduced into the animal by any one of the methods previously described for the purpose. For instance, to obtain expression in a mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include the genes encoding casein (cf. US 5,304,489), beta-lactoglobulin, alpha-lactalbumin and whey acidic protein. The currently preferred promoter is the beta-lactoglobulin promoter (cf. Whitelaw et al., Biochem J. 286, 1992, pp. 31-39).

It is generally recognized in the art that DNA sequences lacking introns are poorly expressed in transgenic animals in comparison with those containing introns (cf. Brinster et al., Proc. Natl. Acad. Sci. USA 85, 1988, pp. 836-840; Palmiter et al., Proc. Natl. Acad. Sci. USA 88, 1991, pp. 478-482; Whitelaw et al., Transgenic Res. 1, 1991, pp. 3-13; WO 89/01343; WO

91/02318). For expression in transgenic animals, it is therefore preferred, whenever possible, to use genomic sequences containing all or some of the native introns of the DNA of interest. It may also be preferred to include at least some introns from, e.g. the beta-lactoglobulin gene. One such region is a DNA segment which provides for intron splicing and RNA polyadenylation from the 3' non-coding region of the ovine beta-lactogloblin gene. When substituted for the native 3' non-coding sequences of a gene, this segment may will enhance and stabilize expression levels of the polypeptide of interest. It may also be possible to replace the region surrounding an initiation codon with corresponding sequences of a milk protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression.

For expression of the DNA of interest in transgenic animals it is operably linked to additional DNA sequences required for its expression to produce expression units. Such additional sequences include a promoter as indicated above, as well as sequences providing for termination of transcription and polyadenylation of mRNA. The expression unit further includes a DNA sequence encoding a secretory signal sequence operably linked to the sequence encoding the polypeptide. The secretory signal sequence may be one native to the polypeptide or may be that of another protein such as a milk protein (cf. von Heijne et al., Nucl. Acids Res. 14, 1986, pp. 4683-4690; and US 4,873,316).

Construction of the expression unit for use in transgenic animals may conveniently be done by inserting the DNA sequence of interest into a vector containing the additional DNA sequences, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA sequence encoding a milk protein and to replace the coding region for the milk protein with the DNA sequence of interest, thereby creating a fusion which includes expression control sequences of the milk protein gene.

The expression unit is then introduced into fertilized ova or early-stage embryos of the selected host species. Introduction of heterologous DNA may be carried out in a number

of ways, including microinjection (cf. US 4,873,191), retroviral infection (cf. Jaenisch, Science 240, 1988, pp. 1468-1474) or site-directed integration using embryonic stem cells (reviewed by Bradley et al., Bio/Technology 10, 1992, pp. 534-539). The ova are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop to term. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny, allowing the development of transgenic herds.

General procedures for producing transgenic animals are known in the art, cf. for instance, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986; Simons et al., Bio/Technology 6, 1988, pp. 179-183; Wall et al., Biol. Reprod. 32, 1985, pp. 645-651; Buhler et al., Bio/Technology 8, 1990, pp. 140-143; Ebert et al., Bio/Technology 6: 179-183, 1988; Krimpenfort et al., Bio/Tecnology 9: 844-847, 1991, Wall et al., J. Cell. Biochem. 49: 113-120, 1992; US 4,873,191, US 4,873,316; WO 88/00239, WO 90/05188; WO 92/11757 and GB 87/00458. Techniques for introducing heterologous DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g. Gordon et al., Proc. Natl. Acad. Sci. USA 77: 7380-7384, 1980, Gordon and Ruddle, Science 214: 1244-1246, 1981; Palmiter and Brinster, Cell 41: 343-345, 1985; Brinster et al., Proc. Natl. Acad. Sci. USA 82: 4438-4442, 1985; and Hogan et al. (ibid.). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WO 88/00239, WO 90/01588 and WO 92/11757; and Simons et al., Bio/Technology 6: 179-183, 1988). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to techniques which have become standard in the art. Injection of DNA into the cytoplasm of a zygote can also be employed.

Production in transgenic plants may also be employed. It has previously been described to introduce DNA sequences into plants, which sequences code for protein products imparting to the transformed plants certain desirable properties such as

increased resistance against pests, pathogens, herbicides or stress conditions (cf. for instance EP 90 033, EP 131 620, EP 205 518, EP 270 355, WO 89/04371 or WO 90/02804), or an improved nutrient value of the plant proteins (cf. for instance EP 90 033, EP 205 518 or WO 89/04371). Furthermore, WO 89/12386 discloses the transformation of plant cells with a gene coding for levansucrase or dextransucrase, regeneration of the plant (especially a tomato plant) from the cell resulting in fruit products with altered viscosity characteristics.

In the plant cell, the DNA of interest is under the control of a regulatory sequence which directs the expression of the DNA sequence in plant cells and intact plants. The regulatory sequence may be either endogenous or heterologous to the host plant cell.

The regulatory sequence may comprise a promoter capable of directing the transcription of the DNA sequence of interest in plants. Examples of promoters which may be used according to the invention are the 35s RNA promoter from cauliflower mosaic virus (CaMV), the class I patatin gene B 33 promoter, the ST-LS1 gene promoter, promoters conferring seed-specific expression, e.g. the phaseolin promoter, or promoters which are activated on wounding, such as the promoter of the proteinase inhibitor II gene or the wun1 or wun2 genes.

The promoter may be operably connected to an enhancer sequence, the purpose of which is to ensure increased transcription of the DNA of interest. Examples of useful enhancer sequences are enhancers from the 5'-upstream region of the 35s RNA of CaMV, the 5'-upstream region of the ST-LS1 gene, the 5'-upstream region of the Cab gene from wheat, the 5'-upstream region of the 1'- and 2'-genes of the T_R-DNA of the Ti plasmid pTi ACH5, the 5'-upstream region of the octopine synthase gene, the 5'-upstream region of the leghemoglobin gene, etc.

The regulatory sequence may also comprise a terminator capable of terminating the transcription of the DNA of interest in plants. Examples of suitable terminators are the terminator of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5 of Agrobacterium tumefaciens, of the gene 7 of the T-

DNA of the Ti plasmid pTiACH5, of the nopaline synthase gene, of the 35s RNA-coding gene from CaMV or from various plant genes, e.g. the ST-LS1 gene, the Cab gene from wheat, class I and class II patatin genes, etc.

The DNA of interest may also be operably connected to a DNA sequence encoding a leader peptide capable of directing the transport of an expressed polypeptide to a specific cellular compartment (e.g. vacuoles) or to extracellular space. Examples of suitable leader peptides are the leader peptide of proteinase inhibitor II from potato, the leader peptide and an additional about 100 amino acid fragments of patatin, or the transit peptide of various nucleus-encoded proteins directed into chloroplasts (e.g. from the St-LS1 gene, SS-Rubisco genes, etc.) or into mitochondria (e.g. from the ADP/ATP translocator).

Furthermore, the DNA of interest may be modified in the 5' non-translated region resulting in enhanced translation of the sequence. Such modifications may, for instance, result in removal of hairpin loops in RNA of the 5' non-translated region. Translation enhancement may be provided by suitably modifying the omega sequence of tobacco mosaic virus or the leaders of other plant viruses (e.g. BMV, MSV) or of plant genes expressed at high levels (e.g. SS-Rubisco, class I patatin or proteinase inhibitor II genes from potato).

The DNA of interest may furthermore be connected to a second DNA sequence encoding another polypeptide or a fragment thereof in such a way that expression of said DNA sequences results in the production of a fusion protein. When the host cell is a potato plant cell, the second DNA sequence may, for instance, encode patatin or a fragment thereof (such as a fragment of about 100 amino acids).

The plant in which the DNA of interest is introduced may suitably be a dicotyledonous plant, examples of which are is a tobacco, potato, tomato, or leguminous (e.g. bean, pea, soy, alfalfa) plant. It is, however, contemplated that monocotyledonous plants, e.g. cereals, may equally well be transformed with the DNA.

Procedures for the genetic manipulation of monocotyledonous and dicotyledonous plants are well known. In order

to construct foreign genes for their subsequent introduction into higher plants, numerous cloning vectors are available which generally contain a replication system for E. coli and a selectable/screenable marker system permitting the recognition of transformed cells. These vectors include e.g. pBR322, the pUC series, pACYC, M13 mp series etc. The foreign sequence may be cloned into appropriate restriction sites. The recombinant plasmid obtained in this way may subsequently be used for the transformation of E. coli. Transformed E. coli cells may be grown in an appropriate medium, harvested and lysed. The chimeric plasmid may then be reisolated and analyzed. Analysis of the recombinant plasmid may be performed by e.g. determination of the nucleotide sequence, restriction analysis, electrophoresis and other molecular-biochemical methods. After each manipulation the sequence may be cleaved and ligated to another DNA sequence. Each DNA sequence can be cloned on a separate plasmid DNA. Depending on the way used for transferring the foreign DNA into plant cells other DNA sequences might be of importance. In case the Ti-plasmid or the Ri plasmid of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, at least the right border of the T-DNA may be used, and often both the right and the left borders of the T-DNA of the Ri or Ti plasmid will be present flanking the DNA sequence to be transferred into plant cells.

The use of the T-DNA for transferring foreign DNA into plant cells has been described extensively in the prior literature (cf. Gasser and Fraley, 1989, Science 244, 1293 - 1299 and references cited therein). After integration of the foreign DNA into the plant genome, this sequence is fairly stable at the original locus and is usually not lost in subsequent mitotic or meiotic divisions. As a general rule, a selectable marker gene will be co-transferred in addition to the gene to be transferred, which marker renders the plant cell resistant to certain antibiotics, e.g. kanamycin, hygromycin, G418 etc. This marker permits the recognition of the transformed cells containing the DNA sequence to be transferred compared to non-transformed cells.

Numerous techniques are available for the introduction of DNA into a plant cell. Examples are the *Agrobacterium* mediated

transfer, the fusion of protoplasts with liposomes containing the respective DNA, microinjection of foreign DNA, electroporation etc. In case *Agrobacterium* mediated gene transfer is employed, the DNA to be transferred has to be present in special plasmids which are either of the intermediate type or the binary type. Due to the presence of sequences homologous to T-DNA sequences, intermediate vectors may integrate into the Ri- or Ti-plasmid by homologous recombination. The Ri- or Ti-plasmid additionally contains the vir-region that is necessary for the transfer of the foreign gene into plant cells. Intermediate vectors cannot replicate in *Agrobacterium* species and are transferred into *Agrobacterium* by either direct transformation or mobilization by means of helper plasmids (conjugation). (Cf. Gasser and Fraley, op. cit. and references cited therein).

Binary vectors may replicate in both *Agrobacterium* species and E. coli. They may contain a selectable marker and a poly-linker region which to the left and right contains the border sequences of the T-DNA of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. Such vectors may be transformed directly into *Agrobacterium* species. The *Agrobacterium* cell serving as the host cell has to contain a vir-region on another plasmid. Additional T-DNA sequences may also be contained in the *Agrobacterium* cell.

The *Agrobacterium* cell containing the DNA sequences to be transferred into plant cells either on a binary vector or in the form of a cointegrate between the intermediate vector and the T-DNA region may then be used for transforming plant cells. Usually either multicellular explants (e.g. leaf discs, stem segments, roots), single cells (protoplasts) or cell suspensions are cocultivated with Agrobacterium cells containing the DNA sequence to be transferred into plant cells. The plant cells treated with the *Agrobacterium* cells are then selected for the cotransferred resistance marker (e.g. kanamycin) and subsequently regenerated to intact plants. These regenerated plants will then be tested for the presence of the DNA sequences to be transferred.

If the DNA is transferred by e.g. electroporation or

microinjection, no special requirements are needed to effect transformation. Simple plasmids e.g. of the pUC series may be used to transform plant cells. Regenerated transgenic plants may be grown normally in a greenhouse or under other conditions. They should display a new phenotype (e.g. production of new proteins) due to the transfer of the foreign gene(s). The transgenic plants may be crossed with other plants which may either be wild-type or transgenic plants transformed with the same or another DNA sequence. Seeds obtained from transgenic plants should be tested to assure that the new genetic trait is inherited in a stable Mendelian fashion. See also Hiatt, Nature 344: 469-479, 1990; Edelbaum et al., J. Interferon Res. 12: 449-453, 1992; Sijmons et al., Bio/Technology 8: 217-221, 1990: and EP 255 378.

The method

Variant cells or colonies on a first surface, usually a plate such as an agar plate, that form a clearing zone around them or become coloured or otherwise detectable as a result of a substance that they secrete (e.g. a peptide, small molecule or protein/enzyme) can be recognized by the colony picker and transferred to a second surface, for example an agar plate, a microtiter plate or another kind of spatial array on a suitable surface (e.g. spatial array on textile cloth). Also, if conditions of the surface or, in the case of a substrate plate, the conditions of its incubation is set up appropriately, only cells survive that secrete a substance that makes the cell producing it viable. Viable cells can then be detected due to formation of colonies and be picked.

In an embodiment of the present invention, picking of the colonies producing the most active substance or producing the highest amount of substance may be obtained as follows: The growth plate may contain a substrate that make the first surface or plate appear "cloudy", and which upon enzymatic activity on that substrate produce clearing zones. The colony picker is then programmed to transfer a portion of the colonies in the clearing zones to the second surface, e.g. another plate or spatial array. Such substrates (and the corresponding enzymes that degrade them) may be skimmed milk (proteases),

starch (amylases), crystal violet and lecithin (phospholipases), phytin (phytases).

In another embodiment of the invention, the turn-over of a substrate into product may generate or remove a coloured, fluorescent or otherwise detectable compound. Examples of substrates which are turned into coloured products (or generate other coloured compounds) upon enzyme-catalyzed modification are: Brilliant Green or Rhodamin Red with Olive oil (substrate for lipase); Congo Red and carboxy methyl cellulose (CMC) (substrate for cellulase).

In yet another embodiment of the invention, the first surface is a growth plate which may contain a toxic compound that is degraded by active enzyme, or it may contain a compound that upon cleavage or ligation to some other compound, creates a nutrient or vitamin beneficial for the growth of the cells or colony that secretes the substance. Accordingly, one aspect of the present invention is to provide a method to select or enrich, in a population, a subset of cells producing a desired enzymatic activity. This enrichment is either based on killing or growth-inhibition of non-enzyme-producing cells, or the selective proliferation of enzyme-producing cells. This can be utilized to screen large libraries of enzyme-producing organisms for an enzyme activity of interest. These libraries can contain variants of one gene, all the genes in a given organism or the library can consist of a collection of different organisms.

The enrichment would typically be performed on solid growth media, where the limitation of diffusion of the toxic compound or the limiting nutrient would prevent cross-feeding between enzyme-producing and non-enzyme-producing organisms. However, if the enzyme in question preferentially is *cis*-acting, *e.g.* through a physical attachment to the producing cell (an intracellular enzyme, an enzyme retained in the periplasmic space in Gram-negative prokaryotes, enzyme is membrane/envelope attached or in other ways anchored to the producing cell), or a diffusional barrier is established around the producing cell, enrichment during planctonic growth is possible.

Two distinct principles can be utilized in order to

achieve this selective enrichment of cells expressing a desired enzyme activity:

One rationale is to utilize a desired enzyme activity to detoxify the growth media and correspondingly allow cells of interest to selectively proliferate. The toxicity could be mediated by an antimicrobial peptide, antimicrobial enzyme, an antibiotic, other antimicrobial substances such as heavy metals, or osmotic stress. The active enzyme would then relieve the toxicity/stress by degrading, hydrolyzing, removing or modifying the toxic compound. Alternatively the enzyme could actively help the cell to get rid of the toxic compound or to repair the damage caused to the cell by the toxic compound.

Another rationale for the selective enrichment is to limit the cells in question of a required compound; *e.g.* an energy source, an essential nutrient, a vitamin, co-factor or compound. The presence of a desired enzyme would result in a selective enrichment of the producing cells by making the essential factor available either through synthesis, degradation of a polymer or through the modification of a precursor. The essential compound could be carbon, nitrogen, vitamins, amino acids or fatty acids.

A special case of the last mentioned rationale takes advantage of auxotrophic strains, requiring for example, one or more amino acids, vitamins, prosthetic groups, co-factors etc. The benefit of using auxotrophic mutants is that the repertoire of available compounds using in a selection is drastically broadened.

Another special case is a system where one essential compound is sequestered by another compound. This sequestering effectively makes the essential compound unavailable. Degradation of the sequestering compound will make the essential compound available. Examples of components in this concept are antibody/essential compound (*e.g.* vitamin or cofactor), Avidin/biotin and siderophore/iron. This sequestering is not necessarily direct; antagonists of *e.g.* an essential biosynthetic route can also be employed. Degradation of this antagonist would allow the cell to acquire or synthesize the essential compound and correspondingly proliferate. An example of this is folic acid, of which an

exogenous source is necessary for many microorganisms. Antagonists of folic acid are *p*-aminosalicylic acid and sulphonamides.

In yet another special case of this enrichment system, the essential compound is reversibly modified in a way that results in the compound loosing its activity. The enzyme in question would act on the modified, essential compound and make it available for the cell allowing it to proliferate.

Examples of useful toxic compounds are lysozyme (is detoxified by degradation by protease) or antimicrobial peptides (protease). Also, toxic compounds can be synthesized that are inactivated by cleavage of a designed bond; for example, if the toxicity of the compound relies on the integrity of an ester bond, this compound may be used to select for active esterases. Other bonds and (enzymes) that may be targeted with this approach include: amide bonds (protease), disulfide bonds (PDI, protein disulfide isomerase).

In yet another embodiment of the invention, a large number of target cells (e.g. fungal cells) may be spread out over the first surface with the potential to grow and become a dense cell layer. Colonies of cells producing a substance that inhibits growth of the target cells will create a clearing zone around them and can therefore be picked as mentioned above (see also example 2). The substance referred to may be for example an antibiotic, an antimicrobial peptide or lysozyme. Alternatively, the plate may contain a layer fixed cells, organelles, cell lysate, or cellular or nuclear or cellular fractions thereof. Then colonies of cells producing the growth inhibiting substance are grown on this layer. Other types of cell layers include but is not limited to spores, mycelial fragments and protoplasts.

In yet another embodiment, a large number of target cells (e.g. fungal cells) may be spread out over the first surface with the potential to grow and become a dense cell layer. The target cells may grow poorly in the absence of a growth-stimulating factor (for example growth hormone). Colonies of cells producing the growth-stimulating factor will stimulate growth of the surrounding target cells and therefore can be recognized and picked from this zone of thicker target cell

layer.

In is contemplated that these embodiments include a set-up where the cells or colonies produce a substance that transforms another substance on the plate into a growth stimulating or inhibiting substance.

All the aforementioned embodiments may be performed with destabilizing agents (urea, detergent, SDS) added, or the temperature may be increased in order to destabilize the substance (i.e., detergent can be included in the growth plate if substances (enzymes) are sought that are stable to detergents). Detergents may be the commercially available detergent products Omo Color, Persil Megapearls, or may be model detergents containing essential components of commercial detergents or may contain one or a few types of molecules, e.g. anionic detergents, non-ionic detergents or cationic detergents.

Examples of target cells which are useful in the aforementioned embodiments may be *Staphylococcus aureaus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Microsporum canis, Micrococcus luteus, Botrytis cinerea, Phytophthora infestans, Saccharomyces cerevisiae* and *Penicillum chrysogenum*. Target cells may also include animal cells for the detection of substances that cause cytotocity, cell proliferation, apoptosis, and/or other changes in cellular phenotype. For example, such target cells may include any neoplastic cells or tissues isolated from a plant, or an animal including a human. Any cell derived from an animal infected by a pathogen, in particular, an intracellular pathogen, such as a virus, a bacterium, fungus, parasite or protozoan, can also be used as a target cell. Primary cell cultures, tissue explants, and permanent cell lines derived from cancer tissues, cancer cells, or infected cells may also be used in the invention. Target cells may also include whole live organisms including protozoa and helminth parasites such as nematodes, e.g. Caenorhapditis elegans. Insects at various stages of development (e.g. Drosophila embryos), or various phases of their life cycles (e.g. larva) may be used as target cells. For example, eggs and larvae of insects which are agricultural pests, or hazards to human and/or animal health

can be used as target cells to screen for insecticides and pesticides.

The target cells may be genetically engineered to produce a target molecule for the substance of interest. The target molecule to be expressed in an indicator cell may be an intracellular protein, a cell surface protein or a secreted protein. Non-limiting examples of target molecules include receptors for hormones, cytokines, neurotransmitters, adhesion molecules, oncogenes, transcription factors, signaling molecules such as kinases and phophatases.

Interaction of the target molecule and the desired compound may produce a detectable signal. Interaction of the target molecule with the desired compound on the cell surface or inside the target cell may initiate or modulate biological signal transduction. As used herein, the term signal transduction is not limited to transmembrane signalling, but includes the various signalling pathways that branch throughout the cell and into the nucleus of eukaryotic cells.

The assay system may comprise a target cell that is genetically engineered to contain a reporter gene construct, a reporter gene. The term reporter gene as used herein refers to any genetic sequence that is detectable and distinguishable from other genetic sequences present in the target cell. The reporter gene sequence encodes a protein that is readily detectable either by its presence, or by its activity that results in the generation of a detectable signal. A reporter gene is used in the invention to monitor and report the presence of a desired activity in the assay system used. Examples of useful reporters are light-emitting reporters such as the Green fluorescent protein (GFP), bacterial luciferase LuxAB, or the luciferase from the firefly *Photinus pyralis*, or other enzymes such as beta-galactosidase, chloramphenicol acetyltransferase (CAT), beta-lactamase, beta-glucoronidase, and alkaline phosphatase.

A reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises a composition that enables and support signal generation by the reporter, *e.g.* substrates and co-factors for reporter molecules that are

enzymes. Such compositions are well-known in the art.

In other embodiments, clones may also be grown in a liquid array, for example a 96 well microtiter plate. The colony picker may then be set up to pick based on a colour reaction, or simply growth or non-growth in the individual well. Also, the colony picker may be set up to pick from microtiter plates instead of an agar plate.

Measurement of a given activity in a colony (e.g. by diameter of clearing zone or colour strength in a chromogenic assay) may be combined with a measurement of amount of total extracellular protein, or specific protein (extracellular and/or intracellular). The ratio is then used as a measure of the specific activity of the substance in that colony, and the colony may be picked based on this ratio.

The following examples illustrate the invention.

EXAMPLE 1

Picking of colonies producing clearing-zones on skim-milk agar plates

150 ml 15-fold diluted LB-medium (J. Sambrook, E.F. Fritsch and T. Maniatis (1989), A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press) containing skim-milk and 6 mg/ml Chloramphenicol is poured into a 22x22 cm plate (Genetix). Bacillus cells expressing various mutants of Savinase, suspended in 0.5-1.0 ml LB-medium, are dispensed onto the agar at a density of 1000-5000 and the cells are spread with glassbeads by shaking. The diluted media ensures a homogenous size distribution of the colonies, which are otherwise of too varied size to be efficiently and accurately picked based on the appearance of a clearing zone. The plates are incubated at 37 or 52°C over night. Growth at 52° gives a surprisingly high level of protease activity early in the growth process. Transformants forming colonies surrounded by clearing zones in the agar are recognized and picked by a fully automated QPix from Genetix.

EXAMPLE 2

Picking of colonies producing an anti-fungal activity, thereby

generating clearing-zones on plates covered with a layer of fungi

A large number (more than 10^5) of fungal cells or spores suspended in 1 ml YPD are plated on a 22x22 cm plate containing YPD or other solid media supporting the growth of the fungi, and incubated at 30°C for 2-5 days. Approximately 500-5000 Bacillus cells, each producing a separate variant of an anti-fungal peptide, in 40 ml 2xYT are added to the plate, and the Bacillus cells allowed to sink down on the agar. The liquid is poured off the plate, and the plates incubated at 30°C for 1-5 days. A colony picker is used to pick Bacillus colonies around which a clearing zone has formed. The clearing zone is an area of less dense growth of the fungal cells.

In an alternative format, 500-5000 Bacillus cells each producing a separate variant of an anti-fungal peptide are suspended in approximately 1 ml LB-medium. The cells are dispensed onto a 22x22 cm agar plate and are spread with glassbeads by shaking. The Bacilli are incubated ON/for 1-5 days allowing for colonies to form. A large number (10^4-10^5) of fungal (*e.g. Botrytis cinerae*) spores suspended in 5 ml of YPD containing low melting agarose are spread over the plate and incubated for 2-5 days at 30°C. A colony picker is used to pick Bacillus colonies around which a clearing zone has formed. The clearing zone is an area of less dense growth of the fungal cells. To allow better identification of clearing zones, the fungal and/or bacterial cells may be stained with strain-specific stains or dyes, or by using other techniques known in the art.

EXAMPLE 3

Picking of colonies producing a substance promoting fungal growth

Approximately 1000-5000 Bacillus cells, each producing a separate variant of a growth promoting substance, and a large number (more than 10^5) of fungal cells, are plated on a 22x22 cm plate containing YPD or other solid media supporting the growth of the fungi, and incubated at 30°C for 2-5 days. A colony picker is used to pick Bacillus colonies surrounded by a dense

layer of fungal cells. As in example 2, fungal and/or bacterial cells may be stained with strain-specific chromophores, in order to better detect fungal growth.

EXAMPLE 4

Picking of colonies producing stable protease on agar plates containing urea and/or SDS

Approximately 1000-5000 B. subtilis cells expressing a library of protease variants are spread out on a 22x22 cm LB agar plate (Genetix), containing 1% skimmed milk, and up to approximately 0.02% SDS and/or up to approximately 0.75 M urea, and incubated overnight at desired temperature (between 30°C and 50°C). Bacillus cells producing heat and detergent stable Savinase variants can be recognized and picked by the colony picker, as colonies producing clearing zones.

EXAMPLE 5

Liquid Colony Picking: Liquid phase re-array of clones expressing active substance for high throughput screening using automated workstations and robotic arms

Any colorimetric assay which can be performed reading between 340nM and 800nM is adaptable to this liquid mode colony picking method with the described set-up. The assay should be in a range determined for the particular system and assay, but generally the reading should be between 0.2 and 2.0 for the wavelength used. For example, once background OD is determined, the following enzyme activities could be measured with, for example, but not limited to, the following substrates. (a) Lipase using para nitophenyl butyrate, (b) Laccase using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS)(c) alkaline protease using N-succinyl-Ala-Ala-pNitoranilide or N-succinyl-Ala-Ala-Pro-Phe-pNitoranilide, (d) peroxidase using ABTS and hydrogen peroxide, (e) Novamyl using a coupled substrate consisting of peroxidase , ABTS, glucose oxidase, the dye Direct Violet 51, maltotriose.

The details of this screen simplifies and optimizes the automation of high throughput screens: i) diluted media can be used in small volumes (20 ml - 40 ml) in 96 well plates whereby

the substrates (e.g. 200 ml) can be directly applied to the growth well, ii) viable cultures can be recovered from the growth well, to which substrate has been added without the need for a duplicate plate, iii) it is possible to do more manipulations of a sample in liquid phase than in solid phase (advantage over colony picking from e.g., agar-plate). For example, if the treatment (e.g., heat treatment) or the assay (e.g., assay at low pH) kills the cells, it is possible in the liquid colony picking approach to split the master plate (growth plate) into two plates, an assay plate and a growth plate. The reaction plate can be exposed to a treatment (e.g., high temperature), and based on the selected criteria, the corresponding positive clone can be picked from the growth plate.

EXAMPLE 6

Protease selection assay/Antimicrobial scavenging system

In this approach, positive protease producing clones is enriched on the basis of their ability to detoxify growth media containing peptide-based antimicrobials; in the present example Protamine.

A protease depleted *B. subtilis* strain (SHA273) was transformed with either no DNA or a protease (Savinase) expressing plasmid (pSX222).

An overnight culture of SHA273 and pSX222/SHA273 was individually diluted to a density of $1x10^3$ cells/ml and plated on agar plates with increasing concentrations of the antimicrobial peptide Protamine (0, 50, 75, 100, 125. 150, 175, 200 μg/ml). Agar plates were incubated overnight at 37 °C and the number of colonies were scored. Numbers are shown in the table below and plotted in figure 1.

The result shows that Protamine inhibits the growth of cells with increasing concentrations. In addition, cells expressing Savinase has a higher survival rate compared to cells expressing no Savinase, in the presence of Protamine. These results show that applying an AMP as selective agent, allows preferential growth of protease producing cells.

	Colony count			% survival	
µg/ml protamine	SHA273	pSX222/ SHA273	µg/ml protamine	SHA273	pSX222/ SHA273
0	54	154	0	100	100
50	52	123	50	96	80
75	41	136	75	76	88
100	41	137	100	76	89
125	35	118	125	65	77
150	26°	127	150	48	82
175	10°	93	175	19	60
200	0	0	200	0	0

° Micro colonies

Though the concept above was exemplified with the antimicrobial peptide Protamine, it is not limited to this antimicrobial alone. A number of other protein-based antimicrobials exist and have been characterized that could be employed. Examples of a-helical antimicrobial peptides are Magainin 1 and 2, Cecropin A, B and P1, CAP18; Andropin; Clavanin A, Styelin C and D, and Buforin II. Examples of cysteine-rich peptides are a-defensins HNP-1, HNP-2 and HNP-3, b-Defensin-12, Drosomycin, g1-Pyrothionin and insect Defensin A. Examples of b-sheet peptides are Lactoferricin B, Tachyplesin I, and Protegrin PG1-5. Examples of peptides with unusual amino acid composition are Indolicidin, PR39, Bac5, Bac7 and Histatin. Examples of peptides with unusual amino acids are Nisin, Gramicidin A and Alamethicin. Another example is the antifungal peptide (AFP) from *Aspergillus giganteus*. Furthermore, to select/enrich for a protease with a given substrate specificity, the nature of the antimicrobial agent would reflect this preference. For example, synthetic antimicrobial peptides in all-*D* configuration would enrich cells expressing a protease capable of degrading peptides in this configuration. Other examples are histidine-rich or proline/argenine-rich antimicrobial peptides, and the antimicrobial peptides mentioned above which contain posttranslationally modified amino acids.

The described approach is not limited to antimicrobial peptides, but can also utilize antimicrobial enzymes including lysozymes, phospholipases, oxidoreductases, laccases, chitinases, glucanases or cellulases.

The enzyme employed to quench the toxicity of the protein-based antimicrobial is not limited to a protease. Many antimicrobials contain essential sulfur-bridges, and their activity is destroyed or diminished by breaking or re-arrangement of these sulfur bridges. This could be mediated by for example reductaseses or protein disulfide isomerases. Other enzymes that could quench the activity of protein-based antimicrobials could be *trans*-glutaminases (coupling glutamate and lysine). If the target protein does not contain lysine and glutamate residues available for cross-linking, another protein or peptide could be added that would supply this missing residue and correspondingly allow the inactivation of the antimicrobial through cross-linking.

Yet other enzymes, *e.g.* oxidoreductases, *e.g.* laccases, peroxidases and haloperoxidases, could be employed to destroy the antimicrobial activity

Finally, the enrichment could be strongly enhanced if the proteinaceous antimicrobial agent also served as the only available carbon and/or nitrogen source, cf. the described rationale of in vivo selection.

EXAMPLE 7

Iturin

Iturins are antibiotics produced by some strains of *Bacillus subtilis*. The structure consists of a heptapeptide sequence closed in a ring with lipophilic β-amino acid with typically 14-16 carbon atoms. This antibiotic is a potent antifungal agent and correspondingly can be employed in the present invention against enzyme-producing fungi and yeasts. It has been shown that Iturins interact with the membranes of sensitive organisms, most likely creating pores leading to efflux of essential cellular compounds and/or compromising the membrane integrity.

Again, microbes producing an enzyme activity that would

allow for the inactivation of the antimicrobial agent, Iturin, would selectively proliferate at the expense of fungi or yeast not producing this activity. Essential determinants for the antimicrobial activity of the Iturin are the heptapeptide itself, which is in the invariant chiral configuration LDDLLDL, an invariant Tyrosine residue within these seven α-amino acids, as well as a lipophilic β-amino acid. Due to the peptide-based backbone of the Iturins, cells secreting a protease with the desired specificity would selectively proliferate.

Cells expressing an enzyme activity capable of *O*-methylating the invariant Tyrosine would also selectively proliferate, as this *O*-methylation has been shown to dramatically decrease the antibiotic activity of the Iturins. Finally, the third major structure in the Iturins, the lipophilic β-amino acid, is of strong interest. The lipophilic nature of the β-amino acid is essential, whereas the exact bonding of the lipophilic structure to the hepta-ring is less critical. This allows for the synthetic or semi-synthetic synthesis of Iturin derivatives that contain a specific chemical bonding between the hepta-ring and the lipophilic structure. The breaking of this artificial bond would separate the lipophilic structure and the hepta-ring and, correspondingly, inactivate the antimicrobial. Designing and synthesizing Iturin derivatives with a given type of bonding would allow the selection of a secreted enzyme that were active on this specific type of bond. Other examples of pairs of chemical bonds and the corresponding enzyme are esters, esterases, nitrils and a nitrilases, phosphates and phosphatases, disulfide bridges and reductases, and various glucosidic bonds and enzymes such as glucanases, amylases, pullulanases and glucosidases.

EXAMPLE 8

Another example is a two-component system; one component is a molecule able to penetrate or mediate a molecular transfer into the cell in question; the other component is an antimicrobial activity that on its own is unable to penetrate the cell and hence exert its antimicrobial activity. The

covalent linking of these two components creates a potent antimicrobial. The exact physical linkage between the components is of no or little importance to the antimicrobial activity of these two connected components, and allows for the creation of chimeras physically linked by a substrate of interest. A cell producing an enzyme that would break/split this linkage would inactivate the two-component antimicrobial and hence allow the cell to selectively proliferate in the presence of the two-component antimicrobial.

As a specific example, one component would be a peptide or protein able to penetrate the cell of interest without being toxic on its own. Examples of this could be specific amphipatic α-helices, or scavenging proteins transported over the cellular membrane and into the cell (*e.g.* siderophores). The toxic component could be an antisense molecule, preferentially the artificial nucleic acid mimic denoted PNA. PNA has superior antisense properties when compared to both DNA and RNA, but is unable to penetrate cells. Other candidates of an antimicrobial agent that is unable to penetrate the cell is the CcdB Gyrase inhibitor.

EXAMPLE 9

As an example of the last mentioned rationale for in vivo selection, protease and non-protease producing clone can be utilized. If these two different cell-types are mixed and spread on minimal media agar plates containing essential trace elements, but without both a carbon and nitrogen source except for a protein or peptide, the protease-secreting clones would preferentially proliferate and form colonies on the plates at the expense of non-protease producing clones. As mentioned earlier, this enrichment could be strengthened if an antimicrobial peptide or protein is utilized as carbon and nitrogen source.

EXAMPLE 10

Colony picking among Aspergillus clones secreting active enzyme

It is a difficult task to make protein libraries in filamentous fungi. One problem is the different morphology and

growth rate of different clones. This gives rise to large and small clones, as well as clones that might be outcompeted by other clones. Assaying fungal transformants of different sizes is also a difficult task due to differences in amounts of expressed enzymes.

These problems can be overcome by growing the individual clones in alginate balls with a high molecular weight (HMW) dextran as carbon source. The HMW dextran lets the slow-growing transformants outgrow, without cross contamination from faster growing transformants. Then the balls are spread out on a flat surface, substrate may be added that give rise to for example colouring of clones producing active substance. Beads containing active colonies are picked on the basis of this signal, and transferred to a spatial array, for example a microtiter plate.

Experimental:

A Polymerase chain reaction, using pAHL (carrying a lipase gene) as the template and 2 pmol/ml of each of the oligo primers #115120 and #134532, designed to amplify the lipase gene, is run under the following conditions: 94°C, 5 min.; 30 cycles of (94°C, 30 sec.; 50°C, 30 sec.; 72°C, 1 min.), and 72°C, 5 min. and a commercial Taq polymerase such as AmpliTaq®, (Perkin-Elmer Corp., Branchburg NJ, USA). For example, the PCR conditions may result in a high rate of error, and therefore a library of Lipase mutants are generated.
Protoplasts of the filamentous fungi strain JaL250 is transformed using 2 mg of pENI1298, which had been digested with Ball and SgrA1 to remove most of the lipase encoding sequence, and 5 mg of the above PCR product. The vector and the PCR fragment are allowed to recombine *in vivo* as described in WO 97/07205.

The protoplasts are washed twice with ST (0.6M sorbitol, 100mM TRIS pH7.0) in order to remove $CaCl_2$. The protoplasts are then re-suspended in an alginate solution (1.5% alginate, 2 % high molecular weight dextran ($5\text{-}40*10^6$ kd), 1.2 M sorbitol, 10 mM TRIS pH7.5). Using a pump the suspension is pumped through a tube ending in a small hole, were small suspension droplets are made. These drops fall down (15 cm) into a shake flask (500ml)

containing 0.2M $CaCl_2$, 1.2 M sorbitol, 10 mM TRIS pH7.5. Droplets of an alginate solution (typically 1-2%) turns into hard balls when they encounter a $CaCl_2$ solution (such as a 0.2 M solution). Therefore, small alginate balls of the size 2.5 mm in diameter are generated by this procedure. The protoplast suspension should be made so that approximately 1 out of 5 balls contains a transformed protoplast, in order to avoid multiple clones in the same ball.

The protoplast-containing alginate balls are grown overnight at 30°C degrees in STC (10 mM $CaCl_2$, 1.2 M sorbitol, 10 mM TRIS pH7.5) in order to regenerate the cell wall. After a couple of washes in steril water to remove sorbitol, the balls are transferred to 1* Vogel media and grown 2-3 days at 30°C degrees. The sole carbon is the dextran in the balls. This prevents cross-contamination from ball to ball, and allows slow-growing transformants to gain reasonable biomass.

The alginate balls are spread out on an agar plate. Then, the balls are soaked in 0.01% para nitrophenol-butyrate, 0.1% Triton X-100, 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, and incubated at room temperature for approximately 5 minutes. Balls containing active lipases will appear yellow, and can be picked by a colony picker, for example a fully automated QPix from Genetix, based on absorbance at 405 nm.

CLAIMS

1. A method of screening a large population of variant cells or cell colonies present on a first surface, which cells or colonies may be capable of producing a useful polypeptide, RNA or small molecule, the method comprising the steps of:

i) on the first surface, subjecting the cells or cell colonies to an assay correlated to a property of the useful polypeptide, RNA or small molecule;

ii) by means of a colony picker, selecting cells having the property from the first surface; and

iii) transferring the selected cells to a second surface.

2. The method according to claim 1, wherein the colony picker is capable of performing at least 3000 transfers per hour.

3. The method according to claim 1, wherein the variant cells or cell colonies are selected from bacterial cells.

4. The method according to claim 3, wherein the variant cells or cell colonies belong to a strain selected from the group consisting of the species *Bacillus alkalophilus*, *Bacillus agaradhaerens*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus clausii*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Streptomyces lividans* and *Streptomyces murinus*.

5. The method according to claim 1, wherein the variant cells or cell colonies are selected from fungal cells.

6. The method according to claim 5, wherein the variant cells or cell colonies belong to a strain selected from the group consisting of the genera *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Eupenicillium*, *Emericella*, *Eurotium*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, *Achlya*, *Candida*, *Alternaria*, *Rhizopus* and *Mucor;* preferably the species

Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus niger, Aspergillus nidulans or *Aspergillus oryzae*.

7. The method according to claim 1, wherein the variant cells or cell colonies are selected from yeast cells.

8. The method according to claim 7, wherein the variant cells or cell colonies belong to a strain selected from the group consisting of the genera *Candida, Kluyveromyces, Saccharomyces, Schizosaccharomyces, Candida, Pichia, Hansehula,* or *Yarrowia,* preferably to the species *Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis, Saccharomyces oviformis, Kluyveromyces lactis, Kluyveromyces fragilis, Hansenula polymorpha, Pichia pastoris Yarrowia lipolytica, Schizosaccharomyces pombe, Ustilgo maylis, Candida maltose, Pichia guillermondii* and *Pichia methanolio*.

9. The method according to claim 1, wherein the useful polypeptide is an enzyme.

10. The method according to claim 9, wherein the enzyme is selected from the group consisting of proteases, cellulases (endoglucanases), β-glucanases, hemicellulases, lipases, peroxidases, laccases, α-amylases, glucoamylases, cutinases, pectinases, reductases, oxidases, phenoloxidases, ligninases, pullulanases, pectate lyases, xyloglucanases, xylanases, pectin acetyl esterases, polygalacturonases, rhamnogalacturonases, pectin lyases, mannanases, pectin methylesterases, cellobiohydrolases, transglutaminases and phytases.

11. The method according to claim 1, wherein the presence of the property of the polypeptide, RNA or small molecule is visually detectable.

12. The method according to claim 11, wherein the presence of the property is detected by a clearing zone on the first

surface.

13. The method according to claim 11, wherein the presence of the property is detected by generation or removal of a coloured substance on the first surface.

14. The method according to claim 11, wherein the presence of the property is detected by generation or removal of a flourescent substance on the first surface.

15. The method according to claim 1, wherein the first surface is a growth plate.

16. The method according to claim 15, wherein the plate is an agar plate.

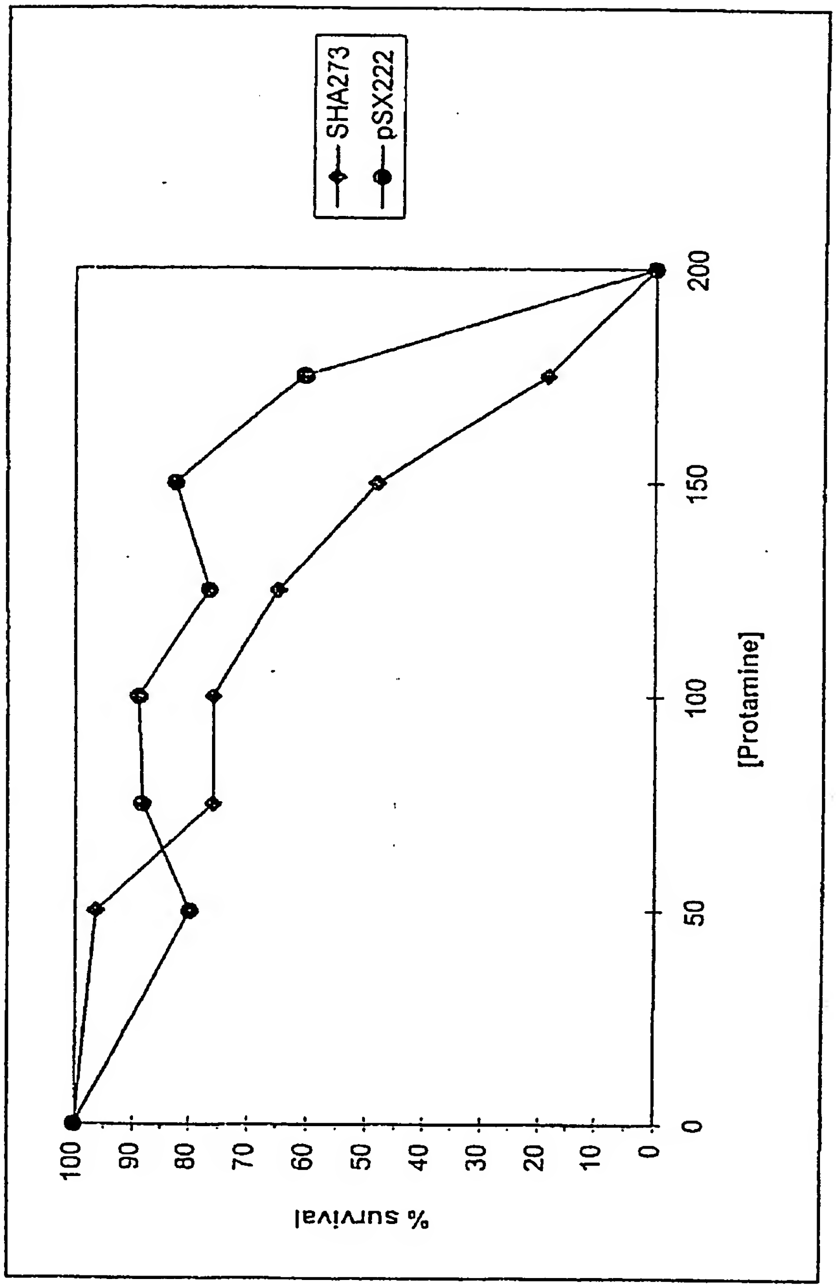
SHA273
pSX222
% survival
[Protamine]
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00565

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2310006 A (GENETIX LIMITED), 13 August 1997 (13.08.97), see page 1, lines 1-12 --	1-16
X	T.A. BROWN, Gene Cloning, An introduction, Second edition, Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, see pages 90-92 -- ----------	1,3-16

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2001	16 -02- 2001

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/DK 00/00565

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2310006 A	13/08/97	GB 9524664 D	00/00/00